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The interaction of ETV6 (TEL) and TIP60 requires a functional histone acetyltransferase domain in TIP60

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Abstract

The ets-family transcription factor ETV6 (TEL) has been shown to be the target of a large number of balanced chromosomal translocations in various hematological malignancies and in some soft tissue tumors. Furthermore, ETV6 is essential for hematopoietic stem cell function. We identified ETV6 interacting proteins using the yeast two hybrid system. One of these proteins is the HIV Tat interacting protein (TIP60), a histone acetyltransferase (HAT) containing the highly conserved MYST domain. TIP60 functions as a corepressor of ETV6 in reporter gene assays. Fluorescently tagged ETV6 and TIP60 colocalize in the nucleus and an increase in nuclear localization of ETV6 was seen when TIP60 was cotransfected. ETV6 interacts with TIP60 through a 63 amino acids region located in the central domain of ETV6 between the *pointed* and the *ets* domain. The ETV6 interacting region of TIP60 mapped to the C2HC zinc finger of the TIP60 MYST domain. The interaction of TIP60 with full length ETV6 required an intact acetyltransferase domain of TIP60. Interestingly, the MYST domains of MOZ and MORF were also able to interact with portions of ETV6. These observations suggest that MYST domain HATs regulate ETV6 transcriptional activity and may therefore play critical roles in leukemogenesis and possibly in normal hematopoietic development.

Keywords: ETV6; TIP60; MOZ; MORF

1. Introduction

Chromosomal rearrangements including balanced translocations and deletions affecting the short arm of chromosome 12 are frequently observed in several types of hematologic malignancies including AML, acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), chronic myelomonocytic leukemia, and chronic myeloid leukemia [1,2]. About half of the balanced chromosomal translocations of 12p result in fusions of ETV6 [3,4]. ETV6 is a member of the ets-family of transcription factors and was cloned as the fusion partner of the platelet-derived growth factor receptor β gene in a t(5;12)(q31;p13) translocation from the leukemic cells of a patient with CMMoL [5].

Until now, more than 19 other partner genes of ETV6 have been identified in hematological malignancies and some solid tumors (congenital fibrosarcoma, mesoblastic nehproma, secretory breast carcinoma). Six of the fusion partners of ETV6 are protein tyrosine kinases (PDGFRB, ABL, JAK2, NTRK3, ARG, SYK, FGFR3, FLT3) [6,7]. The mechanism by which these fusions lead to the development of leukemia seems to be a constitutive activation of the tyrosine kinase function through homodimerization mediated by the *pointed* domain of ETV6 [6]. Retroviral transduction of murine bone marrow demonstrated that the ETV6/JAK2 fusion causes malignant hematologic disease in mice. Transgenic mouse models have shown that the ETV6/PDGFRB fusion causes a myeloproliferative

Abbreviations: GST, Glutathione-*S*-transferase; DTT, dithiotreitol; HSV, Herpes Simplex Virus

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syndrome and the ETV6/NTRK3 and MN1/ETV6 fusion proteins are able to transform NIH3T3 cells [6].

The mechanisms by which the ETV6/CBFA2 fusion or the other reported fusion genes of ETV6 (MN1/ETV6, ETV6/EVI-MDS1, ETV6/BTL, ETV6/ARNT) lead to cellular transformation are less well understood [6]. There are also some translocations involving ETV6 that do not give rise to fusion proteins such as the ETV6/STL and ETV6/ACS2 gene fusions [8,9] or for which it has been shown that the fusion protein is not important for leukemogenesis (e.g. the ETV6/CDX2 fusion) [10,11].

The ETV6/CBFA2 fusion as a consequence of the t(12;21) (p13;q22) is the most common ETV6 fusion found in up to 30% of all childhood B-cell ALLs [12,13]. Interestingly, a high percentage (60–70%) of these leukemias show deletions of the non-rearranged ETV6 allele [14]. ETV6 has also been mapped to the smallest region of overlap of deletions affecting the short arm of chromosome 12 in several hematologic malignancies [15,16]. These findings hint at a possible tumor suppressor function of ETV6.

ETV6$^{-/-}$ mice die at day 10.5 to 11.5 of embryonic development and exhibit defective yolk sac angiogenesis and massive apoptosis in mesenchymal and neural tissue [17]. Experiments using chimeric mice generated with ETV6$^{-/-}$ embryonic stem cells showed that ETV6 is essential for the establishment of adult hematopoiesis in the bone marrow [18]. Recently, it has been shown that disruption of ETV6 causes loss of hematopoietic stem cells in adult bone marrow [19].

In order to arrive at a better understanding of the physiological function of ETV6, which plays such a pivotal role in leukemogenesis and normal hematopoietic development, we used a yeast two hybrid screen to identify proteins that interact with ETV6. The MYST domain histone acetyltransferase TIP60 was identified in our screen and its interaction with ETV6 characterized in detail.

2. Materials and methods

2.1. Plasmid construction

The bait plasmid for the yeast two hybrid screen was constructed by inserting the ETV6 cDNA (obtained from Dr. Golub) in frame into the *Eco*RI cloning site of the pGBT9 (Clontech). This plasmid (pGBT9/ETV6) expresses ETV6 as a fusion gene with the yeast GAL4 DNA binding domain (aa 1–147 of GAL4).

The following portions of ETV6 were cloned in frame with the GAL4 DNA binding domain (aa 1–147) into the eukaryotic expression plasmid pSG424 [72] and/or into the yeast expression vector pGBT9 (Acc #U07646, Clontech): 1) aa 1–652 (full length); 2) aa 1–59; 3) 58–126; 4) aa 1–126; 5) aa 118–345; 6) aa 118–218; 7) aa 219–345; 8) 219–287; 9) 282–345; 10) 249–287; 11) 234–287; 12) 234–317; 13) 219–317; 14) 249–317; 15) 340–420. Construct 6) (aa 118–218) was derived from construct 5) by cutting the insert at the SacI site at position 674 of ETV6.

The following portions of TIP60 (β isoform) were cloned in frame with the GAL4 activation domain into the yeast expression vector pGAD424 (Acc #U07647, Clontech): 1) aa 6–357; 2) aa 148–357 and 3) aa 253–287. The acetyltransferase activity of TIP60β was disrupted by site-directed mutagenesis using Quick Change according to the manufacturer's instructions (Stratagene). The conserved amino acids Gly (aa 380) and Leu (aa 385) in the acetyl coenzyme A binding site of TIP60β were changed into aspartic acid and serine, respectively. The resulting TIP60β-DRS mutant has no acetyltransferase activity (HAT mutant) [20].

The MYST domains of MOZ and MORF were amplified by RT-PCR and cloned in frame into the multiple cloning site of pGAD424 using *Eco*RI and *Sal*I.

The primers used for construction of the different deletion mutants of ETV6 and TIP60 as well as the primers for the amplification and cloning of the MOZ and MORF MYST domains are listed in Table 1. The integrity of all yeast and mammalian expression constructs was verified by sequence analysis.

As a reporter in the cotransfection assays, the plasmid GAL4$_5$tkLUC was used. This plasmid was constructed by replacing the chloramphenicol acetyltransferase gene (CAT) in GAL4$_5$tkCAT [21] with the firefly luciferase gene. This was accomplished by excising the CAT gene from GAL4$_5$tkCAT using *Bgl*II and *Sma*I and replacing it by a *Bgl*II–*Sal*I fragment containing the luciferase gene from pGL3-Basic plasmid (Promega). The resulting construct expresses the luciferase gene under the control of the Herpes simplex virus thymidine kinase promoter.

The pTR334 and pTR89 reporter plasmids were obtained from Scott Hiebert [22]. pTR344 contains the firefly luciferase gene that is under the control of the stromelysin-1 promoter, which has an *ets* and an ETV6 binding site; pTR89 does not contain these binding sites and served as a control.

The pECFP-C1-ETV6 plasmid was constructed by removing the coding sequence of ETV6 from pGBT9-ETV6 with *Bgl*II and *Sal*I and inserting it into pECFP-C1 (Clontech). For pEYFP-N1-TIP60 the complete coding region of TIP60beta including the first 5 codons was amplified by PCR from pGAD-GH-TIP60 using linker primers (N-terminus: 5′-CAT CTC GAG AAG ATG GCG GAG GTG GGG GAG ATA ATC GAG GGC-3′ and C-terminus: 5′-GTA GAA TTC GCC ACT TCC CCC TCT TG-3′) and then inserted into the *Xho*I and *Eco*RI sites of pEYFP-N1 (Clontech). The pCMV-3B-ETV6 plasmid was constructed by inserting the ETV6 cDNA in frame into the *Eco*RI cloning site of pCMV-3B (Stratagene). This plasmid allows the expression of ETV6 with a c-myc-tag in mammalian cells.

2.2. Yeast two hybrid screen

Yeast strain CG-1945 (Clontech) was transformed with the pGBT9/ETV6 bait plasmid using the lithium-acetate procedure. The resulting yeast strain was transformed with a commercial HeLa cDNA library (Clontech) cloned into

Table 1

Primer name	Sequence
ETV6 1T	5′-gactccgaattca t g t ctgagactcctgctc-3′
ETV6 58T	5′-gtggtggaattc c a a tttactggagcagg-3′
ETV6 118T	5′-gactccgaattcc a g c atattctgaagcagag-3′
ETV6 219T	5′-cgttgaattcc a g g gacccaggccgcac-3′
ETV6 234T	5′-ccatgaattcc c t c tgtcagtgtctccc-3′
ETV6 249T	5′-ccatgaattct c c g agtcccacccgaag-3′
ETV6 282T	5′-ccatgaattcc g g c actccgtggatttc-3′
ETV6 340T	5′-gaacgaattcc t g c tttgggattacgtc-3′
ETV6 59B	5′-cctgtgtctagaaa a t t ggctgcaagcgc-3′
ETV6 126B	5′-gctgactctagacga g g t ttcctctgcttc-3′
ETV6 287B	5′-ctgtctagag a a a tccacggagtgccg-3′
ETV6 317B	5′-gctgtctagag t g g ttcatgtaagccag-3′
ETV6 345B	5′-cctgtgtctag a c g taatcccaaagcag-3′
ETV6 420B	5′-ctgctctagac a t a aacctgaacaaaagcc-3′
TIP60 6T	5′-ctctgaattcg a g a taatcgagggctgccgc-3′
TIP60 148T	5′-ctctgaattca a a c ggaaggtggaggtggtt-3′
TIP60 253T	5′-tctcgaattcg a a c tcaccacattgcctgtc-3′
TIP60 287B	5′-cctatgtcgac t g t cgtaggtcacacttggt-3′
TIP60 357B	5′-cctatgtcgacc t t g gagaagtagcccacga-3′
MOZ 511T	5′-gctgaattcg t c a ttgagtttgggaagta-3′
MOZ 708B	5′-gacgtcgacg t c a ttttggtgataaaggc-3′
MORF 426T	5′-gctgaattcc g g t acccttctgtgattga-3′
MORF 632B	5′-gacgtcgag a t g ctgatgtgcctctcat-3′

Primer name: The number indicates the first (in the case of T=top strand primers) or the last (in the case of B=bottom strand primers) amino acid. The codons of the first or last amino acid are underlined in the primer sequence.

pGAD-GH (prey plasmids). Colonies growing on dropout plates lacking leucine, tryptophan, and histidine supplemented with 20 mM 3-amino-1,2,4-triazole (3-AT) were picked for further analysis. The bait plasmid was removed from clones exhibiting a positive beta-galactosidase reaction by overnight growth in YPD medium and subsequent screening of clones on appropriate dropout plates. Only those clones that did not show activation of the HIS3 and LAC-Z reporter genes in the absence of the bait plasmid were analyzed further.

2.3. GST pulldown

The ETV6 cDNA was subcloned into pBluescript IISK (Stratagene) and ^{35}S-methionine-labelled ETV6 protein was prepared from 1 μg of this plasmid using T3 RNA polymerase and a reticulocyte lysate coupled transcription translation kit (Promega) in a 50 μl volume. Prey cDNA inserts were released from pGAD-GH with *Eco*RI and *Xho*I and cloned into *Eco*RI and *Sal*I digested pGEX-4T [73]. GST fusion proteins were expressed in *E. coli* strain BL21 (Stratagene) and purified from bacterial proteins by incubation with glutathione sepharose beads. 25 μl of the transcription translation reaction was incubated with 30 μl of sepharose bound GST fusion proteins or sepharose bound GST as control in 200 μl protein binding buffer (50 mM K-phosphate pH 7.5, 100 mM KCl, 10% glycerol, 0.1% Triton X-100). After extensive washing of the beads with binding buffer the beads were boiled and separated on an 8% SDS-PAGE gel. The dried gel was exposed to X-ray film at room temperature overnight.

2.4. Reporter gene assays

1×10^5 NIH3T3 or HEK293T cells were transiently transfected with a total of 1.7 to 2 μg plasmid DNA in 35 mm dishes using Superfect transfection reagent according to the manufacturer's instructions (Stratagene). 0.1 μg of plasmid pCMV-βGal (Clontech) expressing beta-galactosidase under the control of the CMV promoter or pRL-Null (Promega, Mannheim), a promoterless *Renilla* luciferase plasmid, was included in each transfection as a control. Cells were harvested after 48 h and lysed in 100 μl lysis buffer (100 mM KH_2PO_4 pH 7.8, 0.2% v/v Triton X-100, 0.5 mM DTT). Firefly luciferase, β galactosidase and *Renilla* luciferase activity were assayed on a Berthold LB 592 luminometer (AutoLumat LB953, EG&G Berthold) as described [23]. In cases were *Renilla* luciferase was used as a transfection control, firefly luciferase and *Renilla* luciferase were assayed using the Dual-Luciferase reporter Assay Kit (Promega, Mannheim) [24]. Luciferase activity was normalized to the β galactosidase activity or *Renilla* luciferase. All assays were done at least in triplicate. Trichostatin A (TSA) was added to the culture medium 24 h after transfection at a final concentration of 100 ng/ml.

2.5. Immunoprecipitation

1×10^6 HEK 293T cells were grown in 100 mm dishes and transfected with pCMV-3B-ETV6 and pEYFP-N1-TIP60 as described above. In each immunoprecipitation experiment, 400 μg total cellular lysate was used. Preclearing was performed by incubating the lysate with 30 μl Protein A-Agarose (Roche, Penzberg, Germany) and 2 μg normal rabbit IgG (Santa Cruz Biotechnology, Inc.) in 1 ml binding buffer (10 mM Tris–HCl pH 8, 150 mM NaCl and 0.5% Triton X-100) for 1.5 h. The resulting supernatant was then incubated for 5 h with 2 μg of either specific goat anti-c-Myc antibodies (Santa Cruz Biotechnology, Inc.) or normal goat IgG, which had been previously incubated with 30 μl Protein A-Agarose for 1.5 h. Incubation steps were carried out at 4 °C with gentle agitation. Precipitates were washed 3 times with binding buffer, resuspended in SDS sample buffer and denatured for 5 min at 95 °C.

2.6. Immunoblotting analysis

Immunocomplexes and cellular lysates were electrophoresed on 8% SDS-PAGE and transferred to nitrocellulose membrane (Hybond™ ECL™, Amersham Pharmacia biotech). The membranes were blocked for 1 h with 5% nonfat dried milk at room temperature and probed with mix of goat anti-c-Myc antibodies (Santa Cruz Biotechnology, Inc.) and rabbit anti-GFP (Molecular Probes Europe BV), followed by secondary antibodies conjugated to horseradish peroxidase. Proteins were detected with enhanced chemiluminescence (ECL, Amersham Pharmacia biotech).

2.7. Mapping of interacting domains

To map the protein interaction domains of ETV6 and TIP60, yeast strain CG-1945 was cotransformed with the ETV6 or its deletion mutants cloned into pGBT9 and TIP60 or its deletion mutants cloned into pGAD-GH/TIP60. Growth was assayed on dropout plates lacking leucine and tryptophan (-L, -W: transformation control) or dropout plates supplemented with 20 mM 3-AT lacking leucine, tryptophan and histidine (-L, -W, -H: interaction assay).

2.8. Subcellular colocalization

For subcellular localization studies (Fig. 2A to E and K), NIH3T3 cells were grown on coverslips and transfected with pECFP-C1-ETV6 and/or pEYFP-N1-TIP60 as described above. 48 h after transfection the cells were fixed as described [25] and examined with a Zeiss Axiovert epifluorescence microscope equipped with a CCD camera. Images were captured with the Improvision software (Improvision, Coventry, UK).

For the confocal images (Fig. 2F to I) U2OS cells were grown on coverslips and cotransfected with pCMV-ETV6 and pEYFP-N1-TIP60 using PEI (Sigma). After 24 h cells were fixed with PBS 2% paraformaldehyde for 10 min, permeabilized with PBS 0.1% Triton X for 10 min and blocked with PBS 10% FCS for 1 h. Coverslips were incubated with polyclonal ETV6 rabbit antibodies (Santa Cruz) overnight. Following extensive washing with PBS, Cy3-conjugated secondary antibody (Jackson) was added for 1 h. After further washing steps DNA was stained with DAPI and mounted using Cytomation medium (DAKO). Immunostained specimen were analyzed with a confocal fluorescence laser scanning system (TCS-SP2 scanning system and DM IRB inverted microscope, Leica, Solms, Germany).

3. Results

After screening approximately 1×10^6 prey plasmids, 22 yeast clones were identified that exhibited growth in the absence of histidine and were β-galactosidase positive. After removal of the bait plasmid all 22 clones were negative for β-galactosidase staining. 11 clones were homologous to the human UBC9 gene [26]. The sequence of two clones was homologous to the human HIV1 Tat interactive protein (TIP60) [27]. These two clones were identical to each other and contained almost the complete coding region of TIP60 fused in frame to the GAL4 DNA binding domain from pGAD-GH with the exception of the first 5 amino acids and amino acids 96 to 147. The internal deletion of 52 amino acids from codon 96 to 147 of TIP60 is due to alternative splicing, which has been described as the β isoform of TIP60 [28] (Fig. 1A).

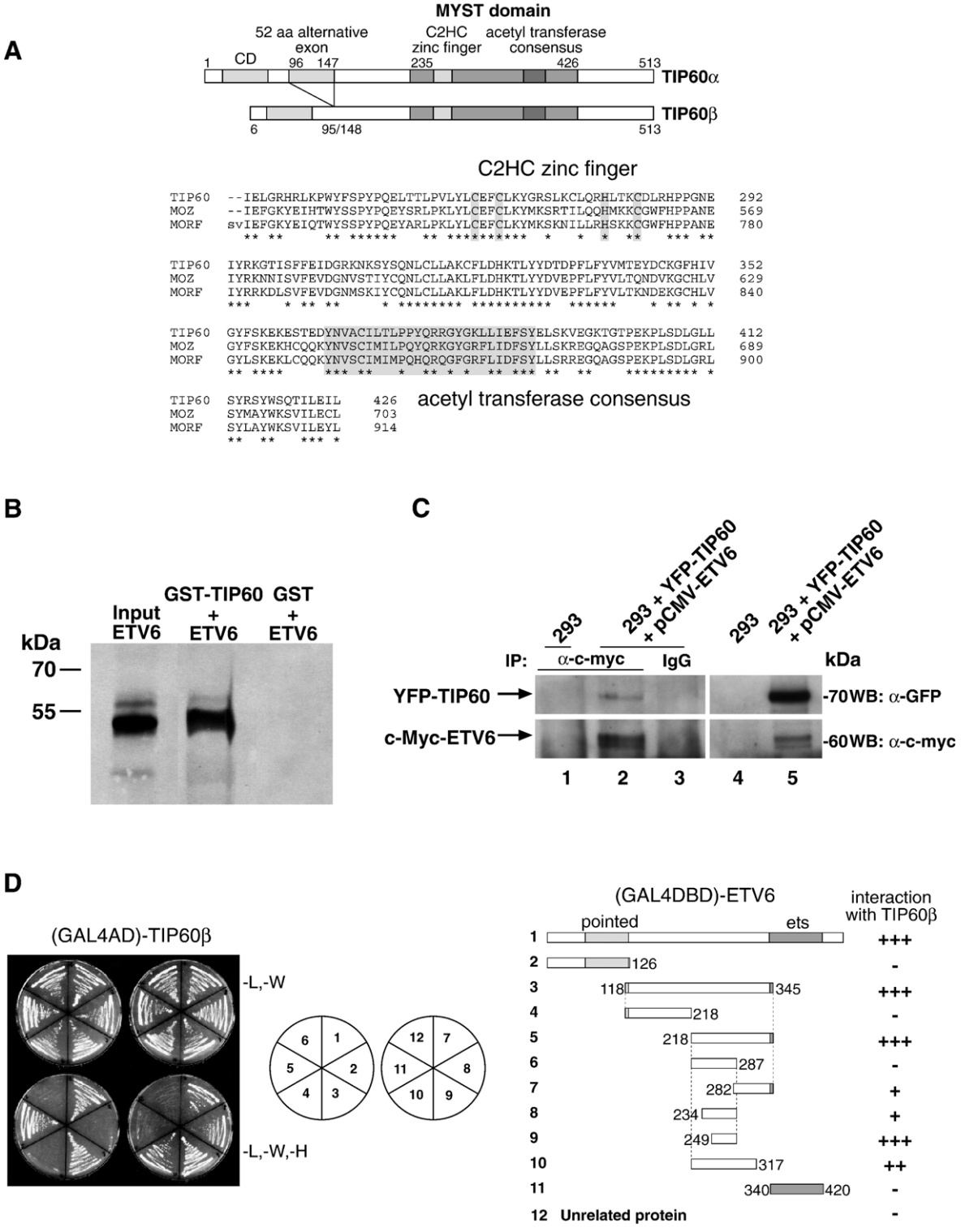
The interaction between TIP60 and ETV6 was verified in vitro by binding of ^{35}S-methionine-labelled ETV6 to GST-TIP60 immobilized on glutathione sepharose beads (Fig. 1B). No binding of ETV6 to the GST-only control is seen.

In vivo interaction was confirmed by coimmunoprecipitation experiments with c-myc tagged ETV6 and TIP60-YFP after coexpression in human epithelial kidney cells (Fig. 1C). In myc-ETV6 expressing cells, TIP6-YFP is coimmunoprecipitated by anti-c-Myc antibodies. In contrast, normal goat IgG does not precipitate either one of the proteins.

A weak positive interaction signal between ETV6 and TIP60 was obtained in the mammalian two hybrid system (2 fold transcriptional activation compared to the strongest negative

control). This could be due to the strong transcriptional repressor activity of ETV6 (data not shown).

To delineate the domain of ETV6 which is responsible for interaction with TIP60, various pGBT9 constructs expressing ETV6 deletion mutants were cotransformed with the pGAD-GH/TIP60 plasmid into yeast strain CG-1945. Interaction between TIP60β and the various portions of ETV6 was assayed by examining the growth of the double transformants on SD, -Leu, -Trp, -His dropout plates (Fig. 1D). The following ETV6 mutants expressed in pGBT9 showed growth on the selection plates indicating interaction with TIP60β: ETV6(118–345), ETV6(218–345), ETV6(282–345), ETV6(234–287), ETV6 (249–287), and ETV6(218–317). The interaction of TIP60 with the ETV6 deletion mutants ETV6(234–287) and ETV6 (218–317) was slightly weaker than with the other deletion mutants. The fact that ETV6(218–287) does not interact with



TIP60 might be explained by the presence of ETV6 domains that block interaction between aa218 and 234 which cannot be properly controlled by TIP60 in these small deletion mutants.

These results show that 38 amino acids of ETV6 between aa 249–287 and the 63 amino acids between aa 282 and 345 are each sufficient for interaction with the full length TIP60β protein.

To further characterize the interaction between TIP60 and ETV6, transient cotransfection experiments of cyan fluorescent protein-tagged ETV6 (CFP-ETV6) and yellow fluorescent protein-tagged TIP60 (TIP60-YFP) were performed in murine fibroblast. When CFP-ETV6 was transfected alone, it showed both nuclear and cytoplasmic localization with varying ratios of nuclear to cytoplasmic CFP-ETV6 in different cells. Some cells showed both nuclear and cytoplasmic CFP-ETV6 (Fig. 2A), while others showed only nuclear CFP-ETV6 (Fig. 2B). The amount of nuclear CFP-ETV6 seemed to stay rather constant. However, in some cells with both nuclear and cytoplasmic CFP-ETV6 there was a much greater amount of cytoplasmic CFP-ETV6 present (Fig. 2B). In single transfections, TIP60-YFP was almost exclusively found in the nucleus (Fig. 2C). Small amounts of TIP60-YFP could be seen in the cytoplasm of some cells when the image was over-exposed (Fig. 2D and E). Coexpression of myc-ETV6 and YFP-TIP60 revealed colocalization of both proteins in the nucleus. In the confocal scans of U2OS nuclei, myc-ETV6 and TIP60-YFP showed a very similar distribution (Fig. 2F, G and H). This was also obvious in a confocal line scan through one of the nuclei where the intensity levels for myc-ETV6 (red) and TIP-YFP (green) showed a similar pattern which was distinct from the curve for the DAPI stain (blue) (Fig. 2K). Interestingly, in the cotransfection experiments the number of cells exhibiting a cytoplasmic CFP-ETV6 distribution decreased and the number of cells with nuclear CFP-ETV6 increased as the ratio of input TIP60-YFP to CFP-ETV6 plasmid was increased (Fig. 2L), suggesting that TIP60-YFP can influence the subcellular localization of CFP-ETV6.

Several deletion mutants of TIP60 were used to identify the protein domain of TIP60 that is responsible for interaction with ETV6. These experiments clearly demonstrated that the 34 amino acids containing the C2HC zinc finger of the TIP60 MYST domain (aa 253–287) are an important interaction domain. Interestingly, this zinc finger is capable of interacting only with two ETV6 deletion mutants which include aa 282–345 of ETV6 (ETV6(218–345) and ETV6(282–345)) but not with the full length ETV6 protein or the deletion mutant containing aa 118–345. The full length ETV6 protein as well as the deletion mutant ETV6(118–345) require the complete TIP60β protein for interaction (Fig. 3A).

Interestingly, the C-terminal deletion mutant of TIP60β (aa 6–357) that lacked the HAT domain of TIP60 was not able to interact with the full length ETV6 protein or the ETV6(118–345) deletion mutant.

To explain this remarkable interaction pattern between the ETV6 and TIP60β deletion mutants, we hypothesized that interaction between ETV6 and TIP60β was a two step process: In the first step a ETV6 might be acetylated by TIP60β which would result in a conformational change of ETV6; then the zinc finger of the TIP60β MYST domain would be able to interact with an exposed interaction interface in the ETV6 central domain. To test this hypothesis, the TIP60β DRS mutant, which lacks acetyltransferase activity, was tested for interaction with ETV6 and several ETV6 deletion mutants in the yeast two hybrid system. Interestingly, the TIP60β-DRS mutant was not able to interact with full length ETV6 or with the central domain (aa 118–345) of ETV6. However, the HAT deficient TIP60β mutant was still able to interact with the C-terminal half (aa 219–345) and the C-terminal quarter (aa 282–345) of the central domain of ETV6 (Fig. 3B). This is exactly the same interaction pattern which was seen with the TIP60β deletion mutants including the mutant containing only the TIP60β MYST domain zinc finger. These observations agree with what would be expected if the HAT activity of TIP60β is needed to change the conformation of ETV6 to allow efficient interaction.

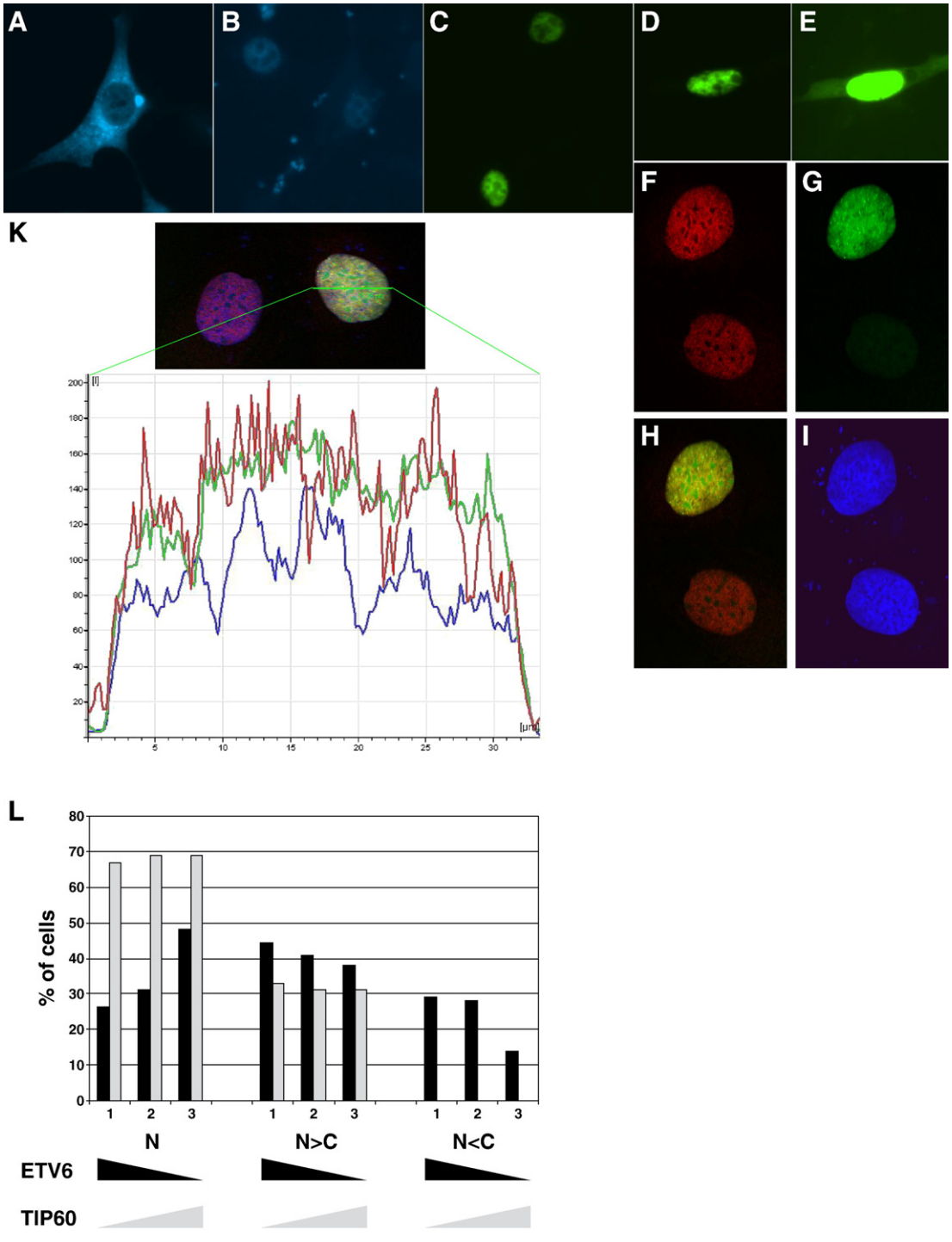
Since the MYST domain of TIP60 shares more than 60% identity to the MYST domains of MOZ (myelomonocytic leukemia zinc finger protein) and to the MYST domain of MORF (MOZ related factor) (Fig. 1A) we tested the interaction of ETV6 and various ETV6 deletion mutants with the MYST domain of these two proteins. As can be seen in Fig. 3C, both the MOZ and the MORF MYST domain interact with ETV6 deletion mutants ETV6(218–345) and ETV6(282–345). These are the same ETV6 deletion mutants that interact with the TIP60 MYST domain zinc finger (Fig. 3A). The MYST domains of

Fig. 1. (A) Diagrammatic representation of the TIP60α and the TIP60β proteins and multiple sequence alignment of the MYST domains of TIP60, MOZ and MORF. The TIP60β form, which was identified in our yeast two hybrid screen, lacks the first 5 amino acids. The position of the MYST domain with the C2HC zinc finger and the acetyl transferase consensus is shown. CD: chromodomain homology. Conserved residues are denoted by an asterisk. (B) GST-Pulldown Assay. Autoradiograph of an SDS polyacrylamide gel electrophoresis after a GST pulldown experiment using ^{35}S-methionine-labelled ETV6 protein and TIP60 fused to GST. Lane 1: 10% ^{35}S-methionine-labelled ETV6 input; lane 2: fraction bound to GST-TIP60 sepharose beads; lane 3: fraction bound to GST-only sepharose beads. (C) Coimmunoprecitation assay. Western blot analysis of an SDS polyacrylamide gel electrophoresis after a coimmunoprecitation experiment using a c-Myc antibody and c-Myc tagged ETV6 protein coexpressed with YFP-tagged TIP60 in 293T cells. The membrane was probed with specific antibodies against GFP (which also recognizes YFP) and c-Myc. Lane 1: Lysate of non-transfected 293 cells immunoprecipitated with c-Myc antibodies. Lanes 2 and 3: Lysate of 293 cells coexpressing YFP-TIP60 and c-myc-ETV6 immunoprecipitated with c-Myc antibodies (lane 2) or IgG (lane 3). Lanes 4 and 5: Loading control with 50 μg of protein extracted either from non-transfected 293 cells (lane 4) and 293 cells coexpressing YFP-TIP60p and c-myc-ETV6 (lane 5). (D) Mapping of the TIP60 interaction domain of ETV6. Yeast clones were cotransformed with pGAD-GH/TIP60 expressing TIP60 as a fusion with the GAL4 activation domain and different deletion mutants of ETV6 cloned into pGBT9 expressed as fusion with the GAL4 DNA binding domain. The upper panel shows the cotransformed clones growing on plates supplemented with histidine (-L, -W), the lower panels depict the growth of the same cotransformants on plates lacking histidine (-L, -W, -H). Growth in the lower panel indicates interaction. The results of these experiments are summarized in the lower half of the figure with diagrammatic drawings of the different ETV6 deletion mutants. The numbers denote the amino acids at the beginning and end of the deletion mutants. The unrelated protein is CALM aa 1–402 fused to the GAL4 DNA binding domain [71].

MOZ and MORF are not able to interact with full length ETV6 or the central domain of ETV6 (ETV6(118–345)) just like the TIP60 MYST domain zinc finger.

It has been shown that ETV6 is a strong transcriptional repressor containing at least two independent repression domains. The *pointed* domain and the central domain (amino acids 118 to 345). The central domain has been shown to recruit the corepressors N-CoR and Sin3. Its repressional activity can be reduced by inhibitors of histone deacetylases like trichostatin A (TSA) [29]. To investigate whether the TIP60 binding domain plays a role in ETV6 repressional activity we performed transient transfection experiments. ETV6 and the above mentioned deletion mutants of ETV6 were expressed as fusion proteins with the yeast GAL4 DNA binding domain (aa 1–147) in mouse NIH3T3 fibroblasts. $GAL4_5$tkLUC was used as reporter plasmid.

ETV6 strongly represses transcription of the luciferase reporter gene in this system. As expected, two domains of ETV6 can be identified that are responsible for this strong transcriptional repression (Fig. 4A). One transcriptional



repression domain is the *pointed* domain (aa 58–126), the other repression domain localizes to the central region of ETV6 (aa 118–345) [29].

We attempted to further narrow down this second repression domain with a series of ETV6 deletion mutants. The ETV6 deletion mutants aa 249–287 and aa 249–317 still mediate transcriptional repression activity which is inhibited by TSA (Fig. 4A). It should be kept in mind, however, that exact mapping of this repression domains might be compromised by incorrect protein folding of the smaller deletion mutants.

The region from aa 282–345 which was shown to interact with the MYST domains of TIP60, MOZ and MORF shows almost no repressional activity. Interestingly, upon addition of TSA this region acts as a transcriptional activator, causing a threefold increase in luciferase activity (Fig. 4A).

Our initial attempts to demonstrate functional consequences of TIP60 coexpression in the described reporter gene assays failed because we were focused on demonstrating relieve of ETV6 repression by TIP60. However, when Nordentoft and Jorgensen reported that TIP60 acts as a corepressor for ETV6 [30], we also noticed a slight corepressor activity of TIP60 for ETV6 (columns 5 and 7 in Fig. 4B). This corepressor activity of TIP60 became very obvious when only the TIP60 interacting domain of ETV6 (aa 282–345) was examined. In the absence of TSA, this region shows slight repressional activity, which is enhanced slightly by cotransfecting TIP60 (9 and 11 in Fig. 4B). In the presence of TSA, ETV6(282–345) shows only a very slight activation of the reporter gene (10 in Fig. 4B). However, the addition of TIP60 results in two fold repression by this region (12 in Fig. 4B).

In order to examine the functional consequences of the ETV6–TIP60 interaction in a more physiological setting, we used a reporter construct (pTR344) in which the luciferase gene is under the control of the ETV6-responsive stromelysin-1 promoter. The pTR344 reporter contains an *ets* and an ETV6 binding site [22]. In transient transfection experiments performed in HEK293T cells, expression of TIP60-YFP led to a slight reduction (1.3 fold) of reporter gene expression (compare columns 1 and 2 in Fig. 5). Expression of CFP-ETV6 led to an approximately 2 fold reduction of reporter gene expression (compare columns 1 and 3 in Fig. 5) and coexpression of both CFP-ETV6 and TIP60-YFP resulted in a 5 fold repression of the reporter gene (column 4 in Fig. 5).

4. Discussion

Analyses of the function of ETV6 as a transcription factor have shown that it acts as a strong transcriptional repressor in transient transfection assays [29,31]. The repressional activity of the central domain (between *pointed* and *ets* domain) of ETV6 is mediated through the recruitment and direct binding of nuclear corepressors (mSin3a, N-CoR, SMRT) [29,32]. The *pointed* domain of ETV6 can also mediate strong transcriptional repression which is not relieved by the addition of TSA [29] and is due to the interaction with the polycomb group protein L3MBTL [33].

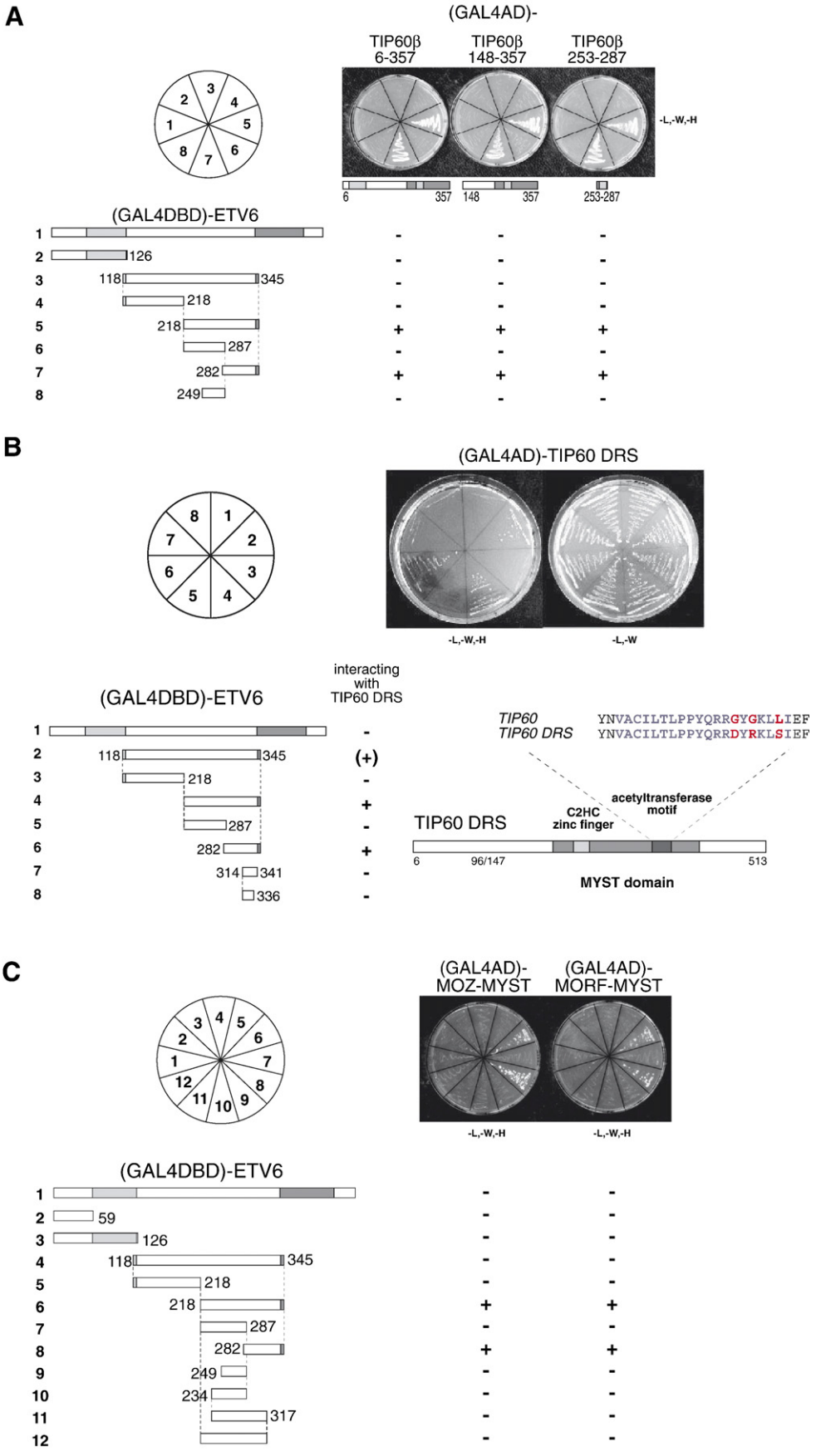
It could be shown that ETV6 is able to dimerize via its *pointed* domain with the ets-family transcription factor FLI1 [34]. In this context, ETV6 is able to repress the transcriptional activity of FLI1 [34]. Recently, it could also be demonstrated that UBC9 can physically interact with ETV6. This interaction relieves transcriptional repression of ETV6 [35] possibly by enhancing nuclear export of ETV6 [36]. ETV6–UBC9 interaction leads to the covalent modification of ETV6 by the small ubiquitin like modifier SUMO-1. Sumoylation of ETV6 seems to be necessary for the cell cycle dependent aggregation of ETV6 in so-called TEL-bodies [37].

To gain further insight into the function of ETV6 we used the yeast two hybrid interaction screen to identify protein interaction partners of ETV6.

One of the interacting proteins was the MYST domain HAT TIP60 (Kamine et al. [27]). The interaction between ETV6 and TIP60 seen in the yeast system could be confirmed by in vitro GST pulldown experiments, by coimmunoprecipitation, through colocalization studies in mammalian cells and in a mammalian two hybrid experiment (data not shown). Unfortunately, due to the low expression levels of the endogenous TIP60 protein in the cell lines tested, a coimmunoprecipitation of the endogenous proteins and therefore a formal proof that the two proteins interact under physicological circumstances, was not possible.

TIP60 was originally isolated in a yeast two hybrid screen using the HIV Tat transactivator protein as a bait. TIP60 acts as

Fig. 2. Subcellular localization of ETV6 and TIP60. NIH3T3 mouse fibroblasts were transiently transfected with CFP-ETV6 or YFP-TIP60 expressing plasmids. (A) Cell with high expression of CFP-ETV6 (300 ms exposure). CFP-ETV6 can be seen in the cytoplasm and the nucleus. Example of cell with more cytoplasmic than nuclear expression of CFP-ETV6 (N<C). (B) Cells with moderate expression of CFP-ETV6 (1000 ms exposure). CFP-ETV6 is localized to the nucleus. (C) Cells with moderate expression of TIP60-YFP (150 ms exposure). TIP60-YFP is localized in the nucleus. (B and C) are examples of cells with only nuclear expression of ETV6 and TIP60 (N). (D) Cell with high expression of TIP60-YFP (80 ms exposure). TIP60-YFP is localized to the nucleus. (E) The same cell as in D but at 700 ms exposure. With this long exposure, a small amount of cytoplasmic TIP60-YFP becomes visible. This is an example of a cell with more nuclear than cytoplasmic expression (N>C). Pictures A through E were acquired using a 63× objective. (F to I) Coexpression of myc-ETV6 and TIP60-YFP in U2OS cells reveals an overlapping nuclear distribution pattern. (F) Two neighbouring cells express similar amounts of immunostained ETV6. (G) The expression of TIP60-YFP is minimal in the lower cell and strong in the upper cell. (H) The merged image of the YFP and Cy3 channels shows that myc-ETV6 and TIP60-YFP have overlapping distribution patterns in the cell nucleus. myc-ETV6 but not TIP60-YFP spares structures that are probably nucleoli. (I) DNA was counterstained with DAPI. (K) Confocal line scan through the nucleus of the upper cell from panels F through I: The picture shows the two cells with all three channels merged. There is a great similarity in the intensity levels of the Cy3 channel for myc-ETV6 (red) and the YFP channel for TIP60-YFP (green) which is distinct from the DAPI intensity (blue). (L) Effect of expression of TIP60-YFP on the subcellular localization of CFP-ETV6 and vice versa. Three independent transfections with varying ratios of CFP-ETV6 to TIP60-YFP expressing plasmid were performed. The total amount of transfected plasmid DNA was kept constant (2 μg). In experiments 1, 2 and 3 CFP-ETV6 to TIP60-YFP ratios of 3:1, 1:1, 7:1 were used, respectively. For each experiment 100 cotransfected cells were examined and the subcellular localization of CFP-ETV6 and TIP60-YFP was scored according to N (nuclear), N>C (more nuclear, but also in the cytoplasm), N<C (less in the nucleus than in the cytoplasm). The percentage of cells in the different categories for each experiment is shown in the bar graph.


A
(GAL4AD)-
TIP60β 6-357
TIP60β 148-357
TIP60β 253-287
-L,-W,-H
6
357
148
357
253-287
(GAL4DBD)-ETV6
1
2
126
3
118
345
4
218
5
218
287
6
287
7
282
8
249
B
(GAL4AD)-TIP60 DRS
-L,-W,-H
-L,-W
interacting with TIP60 DRS
(GAL4DBD)-ETV6
1
2
118
345
(+)
3
218
4
+
5
287
6
282
+
7
314
341
8
336
TIP60
YNVACILTLPPYQRRGYGKLLIEF
TIP60 DRS
YNVACILTLPPYQRRDYRKLSIEF
acetyltransferase motif
C2HC zinc finger
TIP60 DRS
6
96/147
513
MYST domain
C
(GAL4AD)-MOZ-MYST
(GAL4AD)-MORF-MYST
-L,-W,-H
-L,-W,-H
(GAL4DBD)-ETV6
1
2
59
3
126
4
118
345
5
218
6
218
+
7
287
8
282
+
9
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10
234
11
317
12

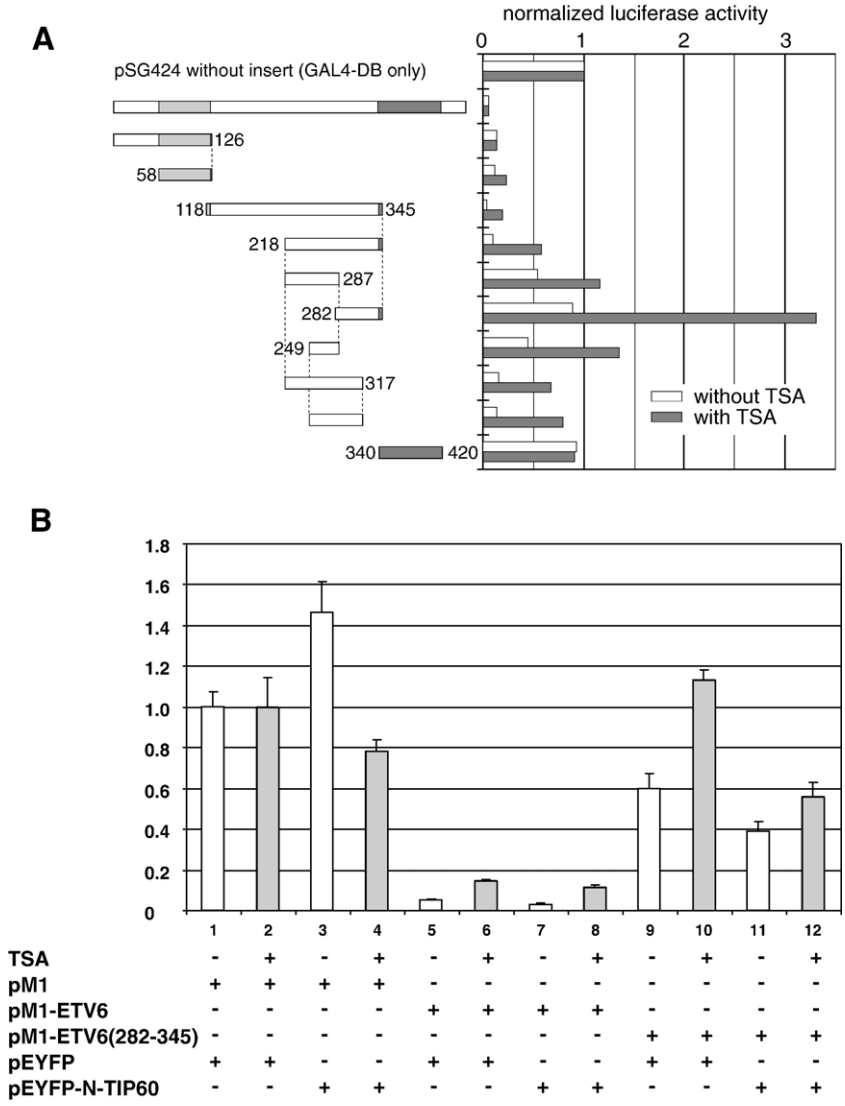

Fig. 4. (A) Mapping of ETV6 transcriptional repression domains and effect of TSA on repressional activity. The effect of a series of ETV6 deletion mutants fused to the GAL4 DBD on a luciferase reporter gene was assayed in transient transfection assays in the NIH3T3 cell line. The luciferase gene was driven by the HSV tk promoter with moderate basal activity ($GAL4_5$tkLUC). All relative light units from the experiments without TSA were normalized to the value obtained when the GAL4DBD was expressed alone. The values from the experiments with TSA were normalized in the same way. The absolute values of the luciferase activity in the experiments with GAL4DBD alone with TSA were about 1.5 fold higher than in the same experiments without TSA. The average of three independent experiments is shown. (B) Influence of TIP60 on the transcriptional activity of ETV6 and the TIP60 interacting domain of ETV6 (aa 282–345). In this assay the same reporter construct ($GAL4_5$tkLUC) as in Fig. 3A was used. Transient transfection was done in HEK293T cells. ETV6 and ETV6(282–345) were expressed as GAL4DBD fusions. TIP60 was expressed as a YFP fusion protein. All odd experiments (without TSA) were normalized to experiment 1. All even experiments (with TSA) were normalized to experiment 2. The average and standard deviation of the normalized luciferase activity of three independent experiments are shown.

a coactivator of HIV Tat [27]. Although there is some debate whether HIV Tat uses TIP60 as a coactivator or whether the interaction with HIV Tat and TIP60 serves to prevent TIP60 from activating genes that could interfere with HIV replication [38]. There are two alternatively spliced forms of TIP60: The full length α form which is localized exclusively in the nucleus and the β form which has both nuclear and cytoplasmatic localization [28]. TIP60β, which lacks 52 amino acids compared to the α form, was found to interact with ETV6 in the yeast two hybrid experiments.

Fig. 3. (A) Mapping of the ETV6 interaction domain of TIP60. The upper panel shows the growth of 24 different yeast cotransformants on plates lacking histidine. Eight different ETV6 deletion mutants fused to the GAL4 DNA binding domain were tested against three different TIP60 deletion mutants fused to the GAL4 activation domain. Each plate assayed all the ETV6 deletion mutants against one of the TIP60 deletion mutants. The results of these assays and the deletion mutants used are shown diagrammatically in the lower half of the figure. (B) Mapping of the ETV6 interaction with the HAT dead TIP60 DRS mutant. The upper panel shows eight different cotransformed clones growing on plates lacking histidine (-L, -W, -H) and on plates supplemented with histidine (-L, -W). The lower panel shows the diagrammatic summary of the results and the point mutations in the acetyltransferase motif of TIP60 DRS. (C) Mapping of the MOZ and MORF MYST domain interaction domain of ETV6. The upper panel shows the growth of 24 yeast cotransformants on plates lacking histidine. The results are diagrammatically summarized in the lower part of the figure.

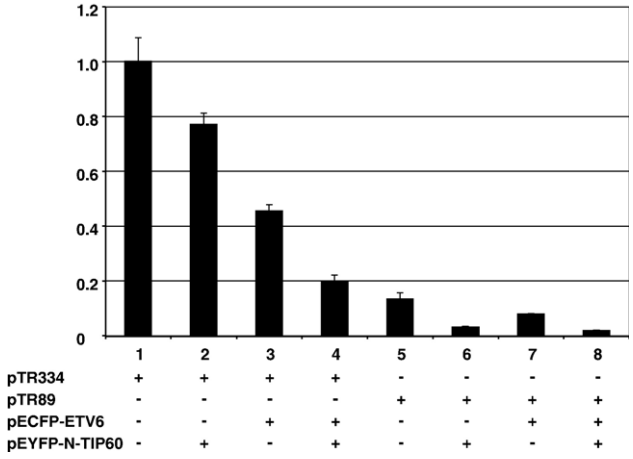

Fig. 5. Effect of coexpression of ETV6 and TIP60 on stromelysin-1 promoter driven reporter gene expression. In this assay the pTR334 and the pTR89 reporter constructs were used in which the expression of the firefly luciferase gene is under the control of portions of the stromelysin-1 promoter [22]. pTR344 contains an ETV6 and an *ets* binding site, pTR89 lacks these two sites and served as a control. ETV6 and TIP60 were expressed as CFP and YFP fusion proteins, respectively, in HEK293T cells. The average and standard deviation of three independent experiments are shown.

The MYST domain comprises more than one third of the amino acid sequence of TIP60. This approximately 200 amino acid long domain derives its name from the four proteins it was originally identified in: *M*OZ, *Y*BF2/Sas3, *S*as2 and *T*IP60. It is an evolutionarily highly conserved, ancient domain [39]. MYST domain proteins can be found in *S. cerevisiae* (Sas2 and Sas3), *C. elegans* and *D. melanogaster* (Mof). The MYST domain consists of a characteristic C2HC zinc finger and an acetyl co-enzyme A consensus binding site/acetyl transferase consensus (Fig. 1A). The N-terminal portion of TIP60 has homologies to chromodomains (Fig. 1A).

TIP60 has subsequently been shown to interact with a number of other – mainly nuclear – proteins. Interestingly, TIP60 can act as a transcriptional coactivator and as a transcriptional corepressor, depending on its interaction partners. It has been shown to interact with the steroid receptors (androgen, estrogen and progesterone receptors), the orphan nuclear receptor RORalpha [40], BCL3, with the intracellular domain of the human interleukin 9 receptor alpha chain, the endothelin receptor A, phospholipase A2 and STAT3 [41]. TIP60 forms a trimeric complex with the intracellular tail of the amyloid precursor protein and the nuclear adaptor protein Fe65 [42] and with STAT3 and HDAC7 [43]. TIP60 enhances transcriptional activity of steroid hormone receptors in a ligand dependent manner and is able to in vitro acetylate distinct lysine residues in the N-terminal tails of histones H2A, H3 and H4 but not of histone H2B [44,45]. The histone acetyltransferase activity of TIP60 is controlled by TIP60 Ser90 phosphorylation via cyclinB/Cdc2 [46]. TIP60 directly acetylates the androgen receptor (AR) which is important for transcriptional activation by the AR [47]. TIP60 represses the transcriptional activity of CREB by direct protein–protein interaction [20] and also represses the activity of STAT3 [43]. Hlubek et al. found that the influence (activation or repression) of TIP60 on the HIV Tat transactivator is cell type specific [48]. It was shown recently that TIP60 is part of a large nuclear multiprotein complex which is involved in DNA repair and apoptosis [49] and that TIP60 is upregulated following DNA damage [50]. Interestingly, acetylation by TIP60 is required for the exchange of histone variants at sites of DNA double strand breaks in *Drosophila* [51]. There is also evidence that TIP60 and P53 might be coregulated through MDM2 and that MDM2 dependent downregulation of TIP60 is one of the mechanisms of MDM2 mediated malignant transformation [50]. TIP60 also interacts with and stabilizes the E3 ubiquitin ligase Pirh2. Pirh2 is a P53 responsive gene and is able to polyubiquitylate P53 [52]. Recruitment of the TIP60 complex was also shown to be important for MYC dependent gene regulation [53], the activation of NFkappaB target genes [54] and further the activation of a metastasis suppressor gene [55].

Recently, Nordentoft and Jorgensen [30] also identified TIP60 as an ETV6 interaction partner and were able to show that TIP60 is a corepressor of ETV6. While Nordentoft and Jorgensen map the TIP60 interaction domain of ETV6 to the *ets* domain and the regions flanking the *ets* domain of ETV6, our results are slightly different. The ETV6 *ets* domain in our hands clearly does not interact with TIP60 (Fig. 1C). However, we map the TIP60 interaction domain of ETV6 to a region just N-terminal of the *ets* domain. Our results allow the interpretation that the *ets* domain is not necessary for ETV6–TIP60 interaction. These discrepancies can probably be explained by the use of different ETV6 deletion mutants in these studies. We could also demonstrate that there are two apparently independent TIP60 full length interaction domains in ETV6, namely from aa 249 to 287 and from aa 282 to 345. These TIP60 interaction domains do not correlate perfectly with the second repression domain of ETV6. It seems that parts of both TIP60 interaction domains are required for maximum repression (Fig. 4A).

Nordentoft and Jorgensen show that mutations in the MYST domain zinc finger abolish interaction with ETV6. Our results show that the CH2C zinc finger on its own is not sufficient for interaction with full length ETV6 but is still capable of interaction with the C-terminal portions of the ETV6 central domain. Putting these results together it can be stated that the CH2C zinc finger of TIP60 is necessary but not sufficient for interaction with full length ETV6. These seemingly contradictory observations suggest a complicated mechanism of interaction between ETV6 and TIP60 which might consist of two events: A first contact between TIP60 and ETV6 leads to a conformational change of ETV6; this exposes the central domain of ETV6 which can then be contacted by the zinc finger of TIP60. The conformational change of ETV6 might be brought about by acetylation of ETV6. We could demonstrate that the TIP60 acetyltransferase function is required for interaction of TIP60 with full length ETV6. A TIP60 acetyltransferase dead mutant failed to interact with full length ETV6 but was still able to interact with the same portions of ETV6 that also showed interaction with the zinc finger of TIP60 (Fig. 3B). These results are compatible with the hypothesis that acetylation of ETV6 by TIP60 is required for their interaction.

However, it should be noted that ETV6 might not be the direct target of acetylation by TIP60 but that another protein in a hypothetical ETV6–TIP60 complex is the target of TIP60 acetylation.

Nordentoft and Jorgensen first reported that TIP60 is a corepressor of ETV6 using both GAL4-DNA binding site domain ETV6 fusions and a reporter plasmid that uses artificial *ets* binding sites. We extend these observations by showing that TIP60 is also a corepressor in the setting of the naturally ETV6-responsive stromelysin-1 promoter. The corepressor function of TIP60 is also evident when only the TIP60 interacting domain of ETV6 (aa 282–345) is used in the GAL4 reporter system (Fig. 4B, experiments columns 9–12). This small region of ETV6 does not mediate transcriptional repression on its own and in fact is able to stimulate transcription when cellular HATs are blocked by TSA (Fig. 4A).

Our colocalization studies in mammalian cells show that TIP60 and ETV6 have a very similar intranuclear distribution pattern, making interaction between these two proteins very likely. Interestingly, higher levels of TIP60 expression lead to more cells that exhibit a predominant nuclear distribution of ETV6 (Fig. 2). Whether this is an artifact of our system or whether higher levels of TIP60 expression lead to a redistribution of ETV6 under certain circumstances (e.g. after UV damage) needs to be examined.

MYST domain HATs have been shown to be involved in a variety of transcription regulatory processes both in gene activation and in gene silencing. The *D. melanogaster* MYST domain protein Mof is required for dosage compensation in the male fly, which is accomplished by increasing the transcriptional activity of the single male X chromosome [39]. The SAS2 and SAS3 proteins in yeast on the other hand are involved in the silencing of genes near the telomeres and the mating type loci [56]. The yeast MYST domain family member ESA1 is essential for growth [57].

HATs are commonly viewed as coactivators of transcription factors, which exert their transcriptional activation function through the acetylation of core histones thereby facilitating gene transcription by loosening nucleosomal packaging. However, there is mounting evidence that histones are not the only targets of acetylation by these enzymes (see acetylation of AR by TIP60) and that not all acetylation of histones inevitably leads to increased transcriptional activity [58,59].

A very interesting aspect of the ETV6–TIP60 interaction is the fact that the human MYST domain HAT MOZ (myelomonocytic zinc finger protein) had originally been identified in a leukemic fusion protein as the partner of CBP in a t(8;16) translocation [60]. Two other fusion partner genes of MOZ have been identified: TIF2 and P300 [61–63]. Recently, a fusion of the MOZ related factor MORF with CBP fusion has been described as the result of a t(10;16)(q22;p13) in cases of acute myeloid leukemia [64].

The fact that the C-terminal fourth of the central domain (aa 282–345) of ETV6 interacts specifically with the zinc finger of the TIP60 MYST domain prompted us to ask whether the MYST domains of MOZ [60] and the related MORF [65] protein would also be able to interact with this region of ETV6. This was indeed the case. Interestingly, the same interaction pattern that was seen between the various ETV6 deletion mutants and the TIP60 MYST domain could also be observed with the MOZ and MORF MYST domains. In light of the results of Nordentoft and Jorgensen, who showed that the C-terminal 34 amino acids of TIP60 are critical for interaction, we would expect that an additional domain (possibly C-terminal to the MYST domain) of MOZ and MORF is required for interaction with full length ETV6. Detailed functional studies of

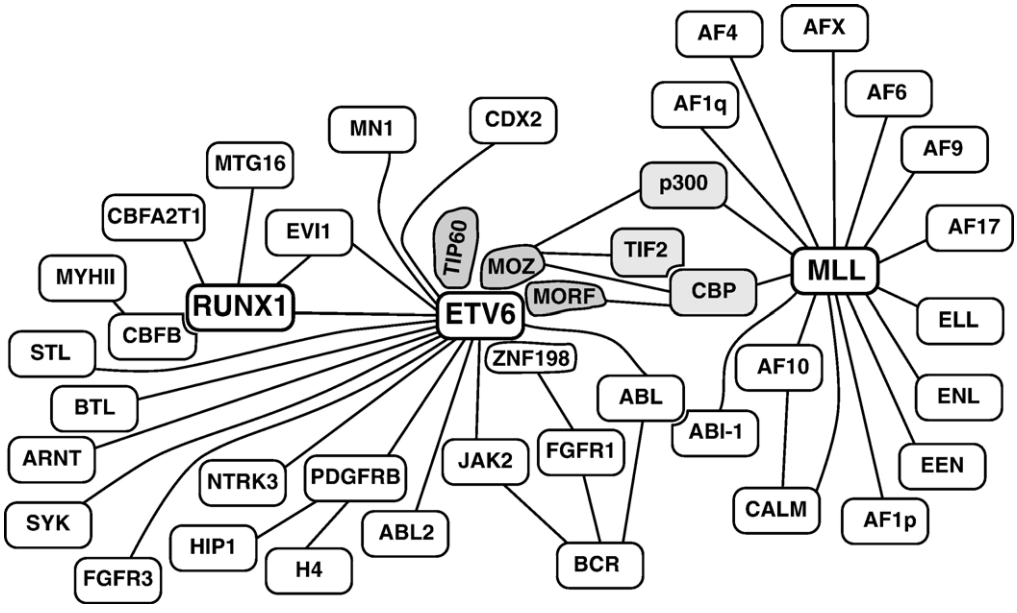

Fig. 6. Diagrammatic representation of leukemic fusion gene network. Genes involved in leukemic translocations are shown in boxes. Boxes connected by a line denote fusion genes. MYST domain HATs are in dark gray boxes, other HATs in light gray boxes. Important protein interactions are shown by boxes touching each other. Note that not all fusions of ETV6 and MLL are shown.

MORF have shown that it contains both a transcriptional activation domain and a transcriptional repression domain. These functions are not dependent on the HAT activity of MORF [65]. It is interesting to note that the C2HC MYST domain zinc finger of MOZ is essential for the transforming properties of the MOZ/TIF2 fusion protein [66]. Deguchi et al. speculate that this might be due to the loss of a putative zinc finger-mediated nucleosomal binding of the MOZ/TIF2 fusion protein. In light of our results, this loss of transforming activity could also be due to the inability of MOZ/TIF2 to interact with ETV6 (or another protein).

The discovery of the interaction between ETV6 and MYST domain HATs links the two large fusion gene networks centered on ETV6 and MLL [67] (Fig. 6). There is thus growing evidence that the alterations of proteins involved in chromatin control like HATs, and especially MYST domain HATs, play an important role in leukemogenesis. In this context, it is quite interesting that Nordentoft and Jorgensen report interaction of ETV6 with ZNF198, a fusion partner of FGFR1 [68,69]. FGFR1 itself has been reported to be fused to BCR in a myeloproliferative disorder [70].

It will require further studies to explore the mechanism of the ETV6–TIP60 interaction and its physiological relevance.

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